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JOURNAL OF VIROLOGY, Apr. 2000, p. 3892–3898 Vol. 74, No. 8
0022-538X/00/$04.00+0

Patterns of Genomic Sequence Diversity among Their Simian Immunodeficiency Viruses Suggest that L'Hoest Monkeys (*Cercopithecus lhoesti*) Are a Natural Lentivirus Reservoir

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Received 9 November 1999/Accepted 25 January 2000

Recently, we described a novel simian immunodeficiency virus (SIVlhoest) from a wild-caught L'Hoest monkey (*Cercopithecus lhoesti*) from a North American zoo. To investigate whether L'Hoest monkeys are the natural host for these viruses, we have screened blood samples from 14 wild animals from the Democratic Republic of Congo. Eight (57%) were found to be seropositive for SIV. Nearly full-length genome sequences were obtained for SIV isolates from three of these monkeys and compared to the original isolate and to other SIVs. The four samples of SIVlhoest formed a distinct cluster in phylogenetic trees. Two of these isolates differed on average at only about 5% of nucleotides, suggesting that they were epidemiologically linked; otherwise, the SIVlhoest isolates differed on average by 18%. Both the level of diversity and the pattern of its variation along the genome were very similar to those seen among isolates of SIVagm from vervet monkeys, pointing to similarities in the nature of, and constraints on, SIV evolution in these two species. Discordant phylogenetic relationships among the SIVlhoest isolates for different genomic regions indicated that mosaic viruses have been generated by recombination, implying that individual monkeys have been coinfected by more than one strain of SIV. Taken together, these observations provide strong evidence that L'Hoest monkeys constitute a natural reservoir for SIV.

Simian immunodeficiency viruses (SIVs) are lentiviruses that have been isolated from a number of species of African primates (1, 3, 11, 13, 21–24, 29, 37, 40, 48). Phylogenetic analyses of those viruses that have been fully characterized have revealed five major lineages (45). Four of these lineages, represented by SIVcpz, SIVsm, SIVagm, and SIVsyk, appear to naturally infect chimpanzees (*Pan troglodytes*) (15, 39, 40), sooty mangabeys (*Cercocebus atys*) (6, 7, 36), African green monkeys (*Chlorocebus* spp.) (1, 2, 8, 11, 13, 23, 27, 29, 37), and Sykes' monkeys (*Cercopithecus albogularis*) (10), respectively. The fifth lineage was initially represented by SIVmnd, isolated from a mandrill (*Mandrillus sphinx*) (48, 49) more than 10 years ago. Much more recently, we described a second member of this lineage, SIVlhoest, isolated from a L'Hoest monkey (*Cercopithecus lhoesti*) (21); while SIVlhoest was found to be quite distant from SIVmnd, nevertheless, the two viruses were clearly more closely related to each other than to any other SIV. Since mandrills and L'Hoest monkeys are not closely related, it seems clear that either SIVmnd or SIVlhoest must have arisen through cross-species transmission.

A number of instances of cross-species transmissions of SIVs have occurred in the past, most notably to humans, but also to other primates in the wild and in captivity, complicating attempts to understand the evolution of the primate lentiviruses. Thus, human immunodeficiency virus type 1 (HIV-1) has been traced to three independent transmissions of SIVcpz from *Pan troglodytes troglodytes* in the western part of central Africa (15, 25, 26, 40), while HIV-2 has resulted from several transmissions of SIVsm in west Africa (5, 7, 17). SIVsm has also been introduced accidentally into macaques in captivity (18, 35), while in the wild, strains of SIVagm have been found in baboons (*Papio* spp.) (28, 51) and a patas monkey (*Erythrocebus patas*) (4). Both members of the fifth SIV lineage, SIVmnd and SIVlhoest, were isolated from animals held in captivity (21, 48, 49), and so it is possible that one (or both) species could have been the recipient of a recent cross-species transmission. To enhance our understanding of the origins and evolution of the primate lentiviruses, we have asked whether L'Hoest monkeys are the natural hosts of this lineage of viruses.

We have taken two complementary approaches to address this question. In the first, we isolated and characterized a lentivirus (SIVsun) from a suntailed monkey (*Cercopithecus solatus*) (3). Suntailed and L'Hoest monkeys are genetically and phenotypically very closely related (19), and SIVsun and SIVlhoest were found to be each other's closest relatives. That result is consistent with host-dependent evolution of SIV within this group of monkeys. The second approach, described here, has been to investigate whether L'Hoest monkeys are indeed a natural reservoir for SIVlhoest by examining sero-

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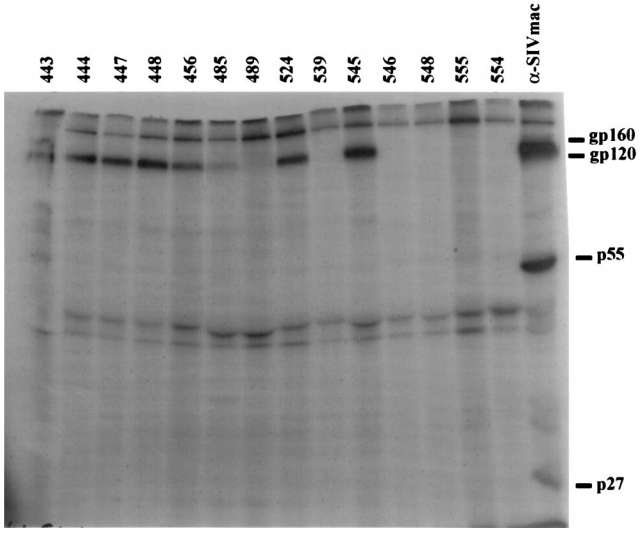

FIG. 1. Radioimmunoprecipitation using SIVmac whole-cell lysate. 443, 444, 447, 448, 456, 485, 489, 524, 539, 545, 546, 548, 555, and 554 are plasma samples from L'Hoest monkeys which were wild caught in the northeastern part of the Democratic Republic of Congo.

prevalence in wild animals and the extent and nature of genetic diversity among SIVs from this species.

For this purpose, blood samples collected in 1988 from 14 wild-caught L'Hoest monkeys (*C. lhoesti*) in the Haut-Congo and Kivu regions of the Democratic Republic of Congo (formerly Zaïre) were investigated for the presence of anti-SIV cross-reactive antibodies. Serology was performed using radioimmunoprecipitation (Fig. 1) with SIVmac251/K6W cell lysate (12). Eight of the 14 monkeys had antibodies cross-reactive with the gp160 envelope glycoprotein of SIVmac, consistent with a seroprevalence of about 57% in this population. One animal (489) which showed a very faint gp160 band was not considered to be SIV seropositive because a repeated test showed a negative result (data not shown). No cross-reactivity was observed with the Gag proteins of SIVmac251 (p55 and p27). Virus was isolated by cocultivation of the human Molt4clone8 cell line with phytohemagglutinin-stimulated peripheral blood mononuclear cells from three of these monkeys (447, 485, and 524), and the viruses were designated SIVlhoest447, SIVlhoest485, and SIVlhoest524, respectively. DNA was extracted from the SIV-infected Molt4-clone8 cells, and four fragments of the genome (*gag*, *pol*, *pol* to *env*, and *env* to long terminal repeat) were amplified by PCR from 500 ng of total cellular DNA. The PCR conditions were 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min per 1 kb of amplified genome, and the following primer pairs were used (underlined sequences represent restriction enzyme sites introduced to facilitate cloning): lhoest *gag*F, 5′ CTAGCTCGAGGCGCCCG AACAGGGACTTCAAG 3′; lhoest *gag*R, 5′ ATTCATTCGA ACTATTGGTCGTCTGGAAAGAG 3′; lhoest *pol*F, 5′ CTAG CTCGAGCTCTTTCCAGACGACCAATAGA 3′; lhoest *pol*R, 5′ ATTCATTCGAAGCACCTTCTCCTTTCCACAGAA 3′; lhoest *pol*-endF, 5′ AGCTCTCGAGTCTGTGGAAAGGAG AAGGTGC 3′; lhoest *env*R, 5′ AGCTTTCGAAGCTGTCA GGCGTGCTTGGAGA 3′; lhoest *env*F, 5′ AGCTCTCGAG TCTCCAAGCACGCCTGACAGC 3′; and lhoest LTR-R, 5′ AGCTTTCGAAAGAGCAGCTGCTTATATGCAG 3′. The resulting fragments were cloned into the pGEM-7Zf plasmid vector (Promega, Madison, Wis.) and sequenced by automated fluorescent sequencing (DNA sequencing kit; Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). The four fragments were assembled to yield an 8.6-kb continuous sequence which spanned all eight genes from the three SIV isolates 447, 485, and 524.

The three new SIVlhoest isolates from Africa were all found to exhibit strong sequence similarity to the previously characterized SIVlhoest7 isolated from a zoo animal. Among the new isolates, two (447 and 485) were very similar to one another, with 90 to 96% sequence identity, depending on the gene product compared (Table 1). All other pairwise comparisons among the four SIVlhoest isolates gave similar and lower identities for any particular protein. On average, the conserved Gag and Pol proteins shared a little under 90% identity, whereas the divergent Tat and Rev proteins were only around 70% identical (Table 1).

Parts of the envelope proteins of the four different SIVlhoest isolates were quite highly conserved, as illustrated in an alignment of the surface unit (gp120) portion of the Env protein (Fig. 2). The four SIVlhoest isolates shared 27 conserved cysteines and 15 conserved N-linked glycosylation sites. The

TABLE 1. Mean percent amino acid identities among SIVs from the SIVlhoest, SIVagm, and SIVcpz lineages

Protein[a]	Intraspecies comparison			Interspecies comparison		
	SIVlhoest[b]	SIVagmVer[c]	SIVcpzPtt[d]	SIVlhoest vs SIVsun[e]	SIVagmVer/Gri/Tan[f]	SIVcpzPtt vs SIVcpzPts[g]
Gag	90 (88–90; 95)	90 (89–92)	80	71	77	68
Pol	88 (86–88; 96)	85 (82–87)	82	73	70	74
Vif	80 (75–80; 93)	72 (68–76)	69	52	57	57
Vpr	90 (88–91; 93)	87 (84–90)	78	63	76	64
Tat	75 (68–76; 92)	67 (64–70)	61	46	57	58
Rev	71 (63–70; 90)	70 (66–78)	50	46	53	54
Env	83 (78–85; 91)	82 (80–84)	67	67	70	51
Nef	85 (82–85; 94)	84 (81–86)	78	56	73	53

[a] Full-length protein sequences (see Fig. 4 for accession numbers) were aligned using ClustalX (47), with minor manual adjustment using SEAVIEW (14).
[b] SIVlhoest isolates 7, 447, 485, and 524. In parentheses, the first two values are the range of identities, excluding the comparison of isolate 447 with isolate 485, which appears as the third value.
[c] SIVagmVer isolates 3, 155, 9063, and Tyo-1. Values in parentheses are the range of identities.
[d] SIVcpzPtt isolates Gab-1 and US. Ptt, *P. troglodytes troglodytes*.
[e] For values, see reference 3.
[f] For values, see reference 21.
[g] SIVcpzGab-1 versus SIVcpzAnt; Pts, *Pan troglodytes schweinfurthii*; for values, see reference 21.

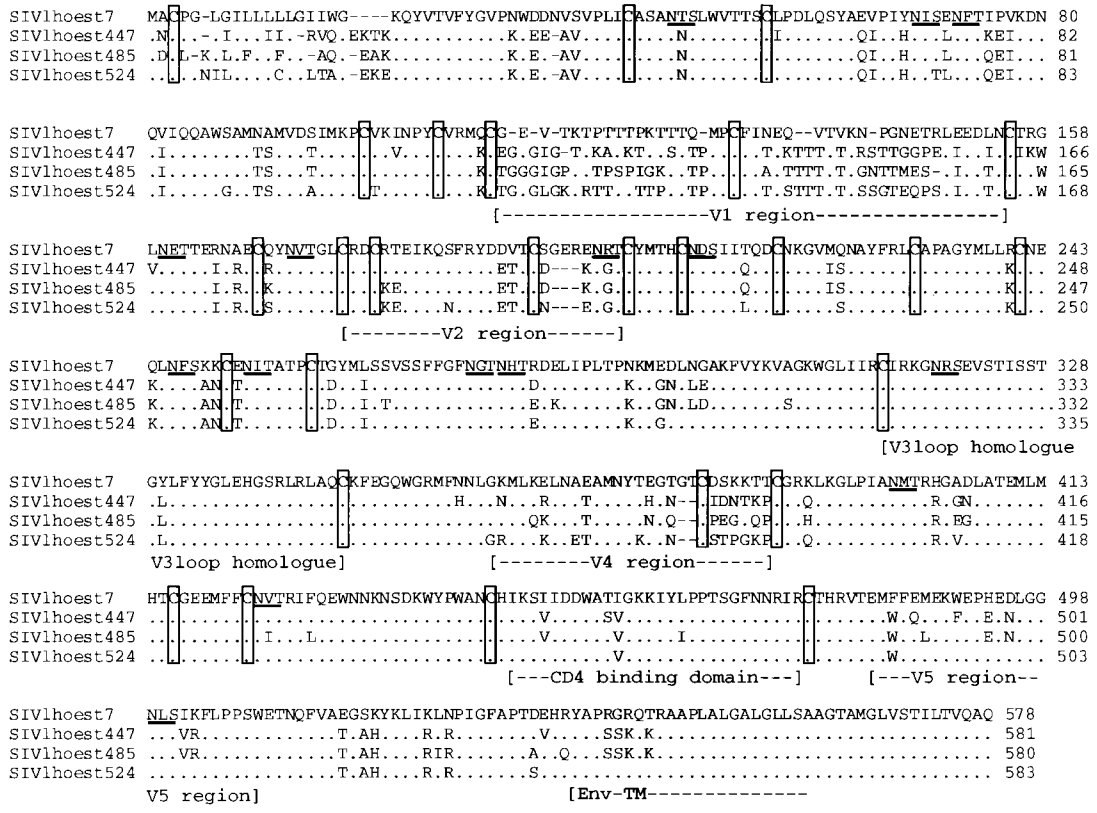

FIG. 2. Comparison of predicted protein sequences of the surface subunit of envelope proteins of SIVlhoest7, SIVlhoest447, SIVlhoest485, and SIVlhoest524. Conserved cysteines are boxed, and conserved N-linked glycosylation sites are underlined. The sequence of the original isolate, SIVlhoest7 (21), is shown on top. Differences, relative to this sequence, in SIVlhoest isolates 447, 485, and 524 are shown aligned below. Dots indicate amino acid identity at a residue, and dashes indicate gaps introduced to optimize alignment. Variable regions analogous to those observed in HIV-1 and other SIVs are indicated, and the cleavage site for the transmembrane glycoprotein (TM) is shown.

amino acid identity was remarkable in the V3 loop homologue and the CD4 binding domain. The V3 loop was totally conserved among SIVlhoest447, 485, and 524 and had only one amino acid exchange in SIVlhoest7. This finding is consistent with the conservation of the V3 loop in other SIV isolates, for example, SIVagm and SIVcpz (27, 37, 50), but in contrast to the V3 loop hypervariability of different HIV-1 isolates (9).

To compare the identities within SIVlhoest isolates with that observed for other SIV strains, the range and mean identities between viral proteins were compared with that between SIVcpz isolates from *P. troglodytes troglodytes* (SIVcpzPtt/Gab-1 and US) and among SIVagm isolates from vervet monkeys (SIVagmVer3, 155, 9063, and Tyo-1) in Table 1. Excluding the comparison between the two closely related SIVlhoest isolates (447 and 485), the extent of protein sequence identity among SIVlhoest isolates was very similar to that among the SIVagmVer isolates. In contrast, the level of identity between the two SIVcpzPtt isolates was always lower than the means for SIVlhoest and SIVagmVer and lower than the minimum identity values for those isolates for all proteins except Vif. As shown in Table 1, the extent of divergence among SIVs isolated from the same species is considerably less than that observed between SIVlhoest and SIVsun, SIVcpzPtt and SIVcpzPts, and SIVagm from different species of African green monkeys (3, 37, 50).

To investigate further the extent of sequence difference across the genome, diversity plots of concatenated gene sequences were constructed (Fig. 3). The close genetic relationship between SIVlhoest isolates 447 and 485 was found consistently across the genome. Two regions of higher divergence between isolates 447 and 485 in the *env* gene were found to also exhibit higher divergence in other comparisons among SIVlhoest isolates (Fig. 3A). The relative extent of sequence difference in comparisons of isolate 447 versus isolates 7 and 524 was found to vary along the genome. For example, in the *env* gene SIVlhoest524 was closer than 7 to 447, whereas in the 3′ half of *pol*, this situation was reversed (Fig. 3A). Such crossing of diversity plots can be diagnostic of mosaic genomes generated by recombination (16). The pattern and extent of nucleotide sequence divergence between SIVlhoest isolates were very similar to those seen between isolates of SIVagmVer. Across most of the genome, the SIVlhoest and SIVagmVer plots tracked each other very closely (Fig. 3B). The simplest interpretation of these data would be that, within each host species, the times since the pairs of viruses last shared a common ancestor are similar. It also suggests that the constraints on sequence evolution of SIV in these two hosts are largely similar. The exceptional regions lie at the 5′ ends of *gag* and *env* and more extensively across the 5′ half of *pol* (Fig. 3B). These unusual areas of the plots might again reflect past recombination. However, the two plots shown were representative of all pairwise comparison plots among SIVlhoest isolates and among SIVagmVer isolates. Thus, it seems more likely that there are some differences in the evolutionary constraints on these regions of the SIV genome in the different hosts. The values in Table 1, based on overall protein comparisons, suggest a uniformly greater level of divergence between

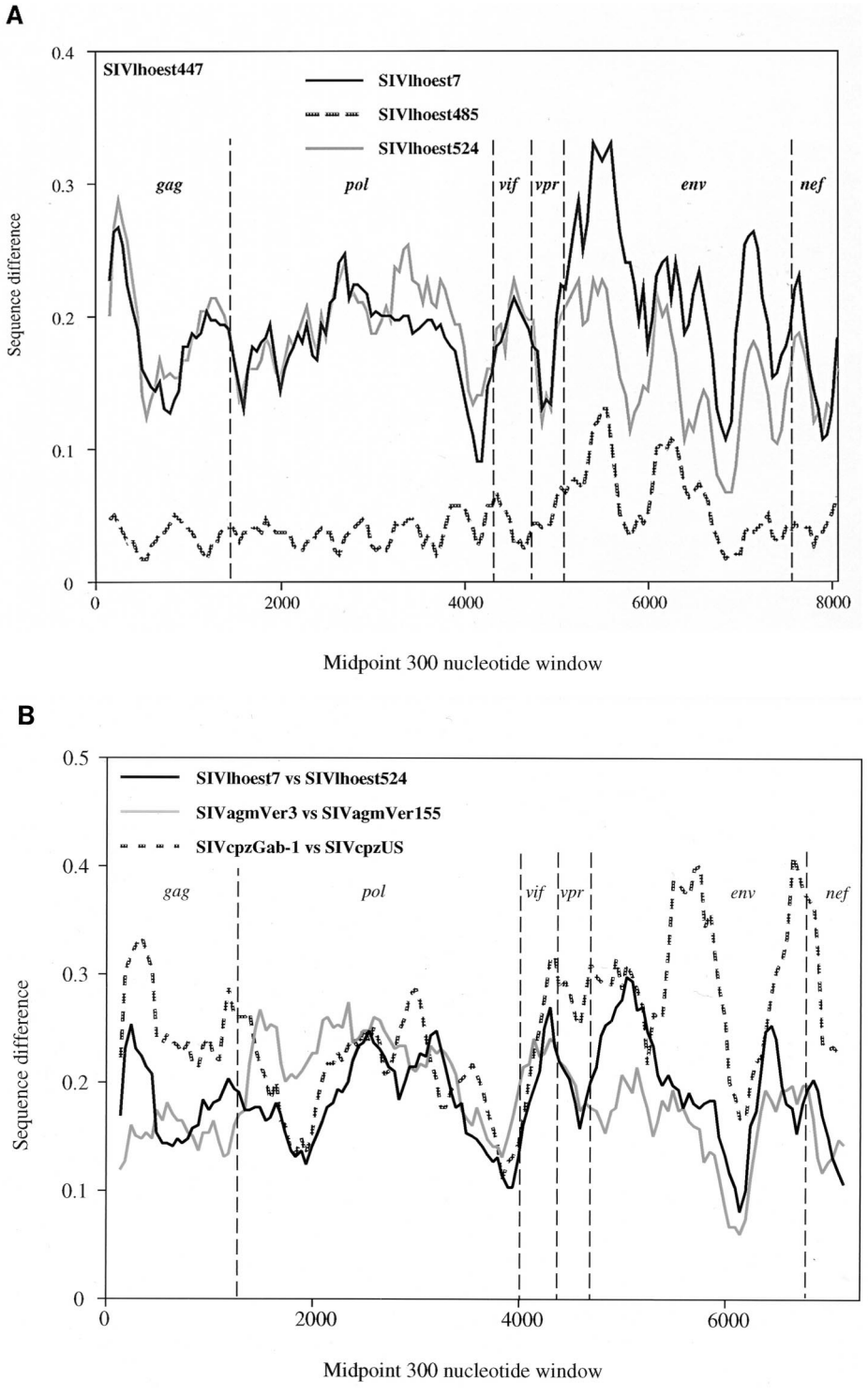

FIG. 3. Diversity plots, showing extent of nucleotide sequence difference along the SIV genome, for windows of 300 nucleotides moved in steps of 50. *gag*, *pol*, *vif*, *vpr*, *env*, and *nef* gene sequences were aligned (based on protein alignments) and concatenated. Regions of gene overlap, as well as regions of uncertain alignment, and sites with a gap in any sequence, were excluded. Comparisons involved SIVlhoest isolate 447 versus isolates 7 (solid black), 485 (dashed black), and 524 (grey) (A) and SIVlhoest isolate 7 versus isolate 524 (solid black), SIVagmVer isolate 3 versus isolate 155 (grey), and SIVcpz isolate Gab-1 versus isolate US (dashed black) (B).

strains of SIVcpzPtt than between strains of SIVlhoest or SIVagmVer compared to the divergence seen in the nucleotide diversity plot (Fig. 3B). Using the latter method, a greater level of sequence difference between SIVcpzPtt isolates was observed only in certain regions of the genome, notably across *gag*, *vpr*, and regions of *env*. Across most of *pol* and *vif*, the SIVcpzPtt strains exhibit a level of divergence similar to that for SIVlhoest strains and SIVagmVer strains.

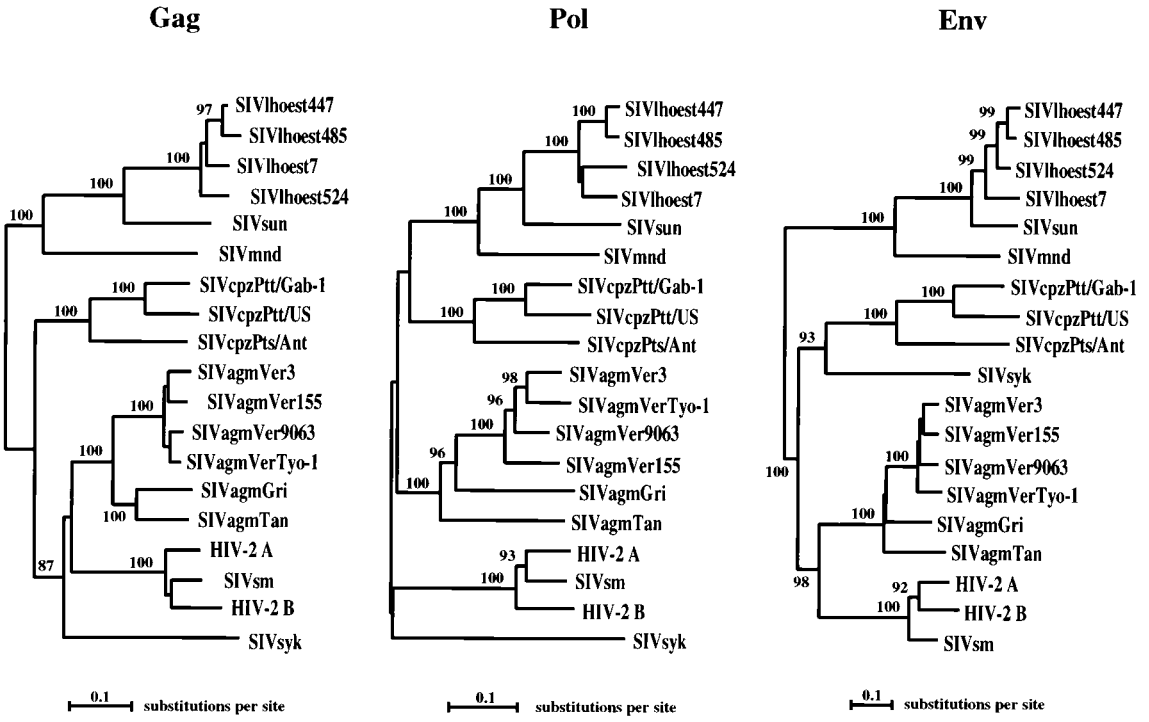

FIG. 4. Phylogenetic relationship of the four SIVlhoest isolates (SIVlhoest7, SIVlhoest447, SIVlhoest485, and SIVlhoest524) to other representatives of the major lentivirus lineages: SIVcpzPtt/Gab-1 (GenBank accession no. X52154), SIVcpzPts/Ant (accession no. U42720), SIVcpzPtt/US (accession no. AF103818), SIVsmPBj (accession no. M31325), HIV-2 subtype A (ROD; accession no. M15390), HIV-2 subtype B (EHOA; accession no. U27200), SIVagmVer3 (accession no. M30931), SIVagmVerTyo-1 (accession no. X07805), SIVagmVer9063 (accession no. L40990), SIVagmVer155 (accession no. M29975), SIVagmGri (gri-1; accession no. M58410), SIVagmTan (tan-1; accession no. U58991), SIVsyk (173; accession no. L06042), SIVmnd (GB1; accession no. M27470), SIVlhoest7 (accession no. AF075269), and SIVsun (accession no. AF131870). The trees were derived by the neighbor-joining method (44) applied to protein distances estimated using Kimura's correction (33) with 1,000 bootstrap replicates, implemented using ClustalX (47). The trees were midpoint rooted. Bootstrap values greater than 80% are shown. Trees derived by maximum-likelihood analysis implemented with PROTML (MOLPHY, version 2.2, J. Adachi and M. Hasegawa, Institute of Statistical Mathematics, Tokyo, Japan, 1994) using the Jones-Taylor-Thornton model (30) with data frequencies differed in no significant way. Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacements per site.

The phylogenetic relationships of the newly derived sequences were estimated by both neighbor-joining and maximum likelihood analyses of Gag, Pol, and Env proteins. Since both methods generated similar tree topologies, only the neighbor-joining results are shown (Fig. 4). The SIVlhoest strains, SIVsun, and SIVmnd formed a lineage-specific cluster with the SIVlhoest isolates being clearly more related to one another than to SIVsun. As expected, SIVlhoest isolates 447 and 485 were found to be closely related in all three trees. However, the branching order among isolates 7 and 524 and the 447-485 cluster differed depending upon the protein used. Thus, SIVlhoest7 clustered with isolates 447 and 485 in the Gag tree but with isolate 524 in the Pol tree and was the outgroup among the SIVlhoest isolates in the Env tree. This discordance among the topologies of the trees was again suggestive of recombination during the evolution of SIVlhoest.

To localize putative crossover points and evaluate the statistical significance of the evidence for recombination among the SIVlhoest lineages, informative site analysis (42, 43) was performed on a four-sequence alignment of the concatenated proteome. Isolate 485 was taken as representative of the 447-485 cluster, and SIVsun was used as a close outgroup. Informative sites (where two of the sequences shared a common residue and the other two shared another) were defined as either type 1, 2, or 3, depending on the branching order that they supported (Table 2). The linear distribution of 92 such

TABLE 2. Regions of the SIVlhoest concatenated proteome, supporting alternate phylogenetic trees

Region	Part of proteome[a]	Coordinates[b]	Informative sites (type 1-2-3)[c]	Tree[d]
1	Gag-Pol-Vif	1–1589	19-9-12	1
2	Vpr-Env	1617–2061	1-3-16	3
3	Env	2109–2203	0-6-0	2
4	Env	2236–2491	2-2-13	3
5	Nef	2494–2693	6-1-2	1

[a] The region labels are approximate, i.e., the breakpoints are not exactly between the proteins.
[b] Amino acid residues of the concatenated proteome.
[c] Numbers of sites of types 1, 2, and 3, each supporting an alternative relationship among SIVlhoest isolates 7, 485, and 524, with SIVsun as an outgroup. Type 1 sites cluster isolates 7 and 485, type 2 sites cluster isolates 7 and 524, and type 3 sites cluster isolates 485 and 524.
[d] Majority type of informative site and inferred tree (see above).

sites along the proteome was mapped, and potential breakpoints between adjacent informative sites were identified as those maximizing the discrepancy, across the breakpoint, of numbers of sites supporting alternative trees. The magnitude of the discrepancy was assessed by heterogeneity chi-square values, and the significance of these values was assessed by permutation tests. These analyses yielded four significant breakpoints, defining five genomic regions (Table 2). For example, the fourth breakpoint was located near the border between the concatenated Env and Nef proteins. This separated a region comprising much of the 3′ half of the Env protein where the majority of informative sites were of type 3, indicating a clustering of isolates 485 (and 447) and 524 as seen in the Env tree (Fig. 4), from a region comprising the Nef protein where the majority of sites were of type 1, supporting a clustering of isolates 7 and 485 (and 447) as seen in the Gag tree (Fig. 4). Most of the informative sites across the proteome were of types 1 and 3, supporting these two alternative trees. However, a region was found in the middle of Env where six adjacent informative sites were of type 2, supporting the final alternative tree in which isolates 7 and 524 clustered.

Within any genomic region where the evolutionary history has not been disrupted by recombination events, the numbers of informative sites supporting the two incorrect trees should be small. This was observed for regions 2 to 5, but in the first region 21 out of 40 informative sites supported trees other than the inferred topology. Recombination between primate lentiviruses typically involves multiple crossovers along the genome (16). Region 1 was very long, covering more than half of the genome, and the three types of informative sites appeared to be nonrandomly distributed across region 1. Several putative breakpoints were identified within this region, but not with statistical significance. One region comprising much of Pol contained mainly sites of types 2 and 3, perhaps explaining why the tree for the Pol protein (Fig. 4) was dissimilar to that for Gag, even though both Gag and Pol were included within region 1 (Table 2). This informative site analysis supported the suggestions from diversity plots (Fig. 3) and the phylogenetic analyses (Fig. 4) that the SIVlhoest sequences reflect a complex history of recombination in the past. This implies that individual monkeys have been coinfected with two or more divergent strains of SIVlhoest, which is not surprising if the evaluation of the seroprevalence in our small sample collection can be extrapolated to a larger L'Hoest monkey population in their natural habitat.

There is considerable precedent in the literature for recombination among the primate lentiviruses. For example, recent recombinants have been identified between different subtypes of HIV-1 group M (16, 43) and between groups M and O (41, 46) in populations where these different clades cocirculate. Discordant branching patterns for strains of SIVsm in trees derived from *gag* and *env* sequences suggest that recombination has occurred among SIVsm in feral sooty mangabeys (7). The discordant branching orders for the four strains of SIVagmVer in Gag, Pol, and Env trees (Fig. 4) may be the consequence of past recombination, and indeed it would be surprising if further investigations of SIVagm do not reveal additional examples.

In conclusion, this study presents evidence that L'Hoest monkeys are indeed the natural reservoir for SIVlhoest. The seroprevalence of SIV among wild L'Hoest monkeys was high, similar to that observed in wild African green monkey populations (20, 31, 32, 34, 38). The four SIVlhoest isolates so far characterized formed a phylogenetic cluster, most closely related to SIVsun from a closely related species of monkey (3). The extent of genetic diversity observed among the SIVlhoest isolates was very similar to that seen previously among SIVagm isolates from naturally infected vervet monkeys (2, 8, 13, 29). Furthermore, the pattern of this genetic diversity across the genome was also largely similar to that in SIVagmVer, especially in terms of the relative conservation of the V3 loop of the Env protein. Finally, evidence that SIVlhoest sequences have undergone recombination is consistent with a high rate of infection in wild populations.

Nucleotide sequence accession number. The sequences of SIVlhoest447, SIVlhoest485, and SIVlhoest524 have been submitted to GenBank under accession no. AF188114, AF188115, and AF188116, respectively.

We thank Malcolm Martin for continued support of this study.

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